LETHALITY AND THE BIOLOGICAL EFFECTS OF X-RAYS IN PARAMECIUM: RADIATION RESISTANCE AND ITS VARIABILITY

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It has been known for a long time that *Paramecium* and certain other Protozoa are able to survive exceedingly high dosages of x-rays (see review, Wichterman, 1953). With low, sub-lethal dosages, paramecia become perceptibly accelerated. In normal bacterized culture media, dosages of 200,000 roentgen (r) and above usually retard motility in *Paramecium*, and there are generally no survivors above 510,000 r. Occasionally, survivors of this high dosage produce clones which, after overcoming irradiation effects, reproduce and flourish in a manner comparable to controls (Wichterman, 1948). X-ray survival curves for microorganisms as reported in the literature vary considerably, apparently depending upon the conditions employed for irradiation. We find, for instance, that with certain methods and under certain conditions in the irradiation of *Paramecium caudatum*, the LD 50—that dosage which results in the death of 50 per cent of irradiated organisms—may vary from 75,000 r to 350,000 r.

The purpose of the present investigation was to establish a standard, repeatable method of irradiation and to analyze the causes of radiation resistance and variability in *Paramecium*.

To fully appreciate the insensitivity of paramecia to x-radiation, we need only examine the LD 50 dosages of other organisms. According to Lea (1947), the 50 per cent survival dosage for yeast is 30,000 r; for the bacterium *B. coli*, 5600 r, and for spores of *B. mesentericus*, 150,000 r. For the algae *Chlorella*, *Ankistrodesmus*, and *Chroococcus*, the LD 50 is 22,000 r, 11,000 r, and 9,000 r, respectively (Bonham and Palumbo, 1951). In this connection, it is to be noted that bacteria in culture fluid, as well as those in the body of *Paramecium* and the symbiotic *Chlorella* in *Paramecium bursaria*, can be destroyed by x-rays without killing the paramecia (Wichterman, 1948). It is thus possible to sterilize such cultures to yield species-pure clones of *Paramecium* as well as colorless races of the normally green species, *Paramecium bursaria*. The recent accounts given by Curtis (1951) and Nickson (1952) for some vertebrate animals commonly used in the laboratory are seen to vary, but relatively low dosages of x-rays are required to produce 50 per cent lethality. For instance the LD 50 for "baby" rats is given as 510 r but 590–1280 r for adults. The LD 50 for other animals follows: mice, 400–840 r; guinea

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pigs, 200–310 r; rabbits, 790–1500 r; dogs, 300–335 r; monkeys, 500 r. According to Sparrow and Rubin (1952), it has been estimated that the LD 50 for man would be approximately 400 r when the x-radiation is received over the whole body in a fairly short period of time. It is therefore worthy of note that *Paramecium caudatum*, with an LD 50 of approximately 340,000 r when irradiated in Nylon syringes, has a radiation resistance 850 times as great as that of man and some common vertebrate laboratory animals.

As a test animal for the evaluation of irradiation effects and associated phenomena, *Paramecium* has many useful features. Beginning with a single specimen, it is possible to obtain for experimentation a genetically uniform, pedigreed strain of enormous numbers of paramecia. This allows for speed and precision of observation generally impossible with other test animals. In addition to being a completely isolated cell, *Paramecium* is a structurally complex organism; hence morphologic changes as a result of irradiation can be determined readily. Irradiation effects are manifested in loss of motility, which may include a change in ciliary action or its complete cessation, dysfunction of contractile vacuoles, change in rate of cyclosis, vacuolization, blistering of the pellicle, changes in body shape, and finally disintegration of the body. Also the division rate, which is an index of vitality, can be compared with the control specimens and expressed in quantitative terms. Additional advantages in x-radiation experiments with paramecia may lie in the field of biochemistry, especially in regard to the effects on respiratory mechanisms which appear to be greatly involved.

Materials and Methods

In the present study, all irradiation work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts. The x-ray generator operates simultaneously two water-cooled Coolidge tubes in alternate parallel. One tube was mounted rigidly on a platform on the floor, and the other tube was supported on a counter-balanced arm which allowed it to be moved vertically and in line directly over the fixed tube. Paramecia in irradiation chambers were thus cross-fired from above and below. The x-ray tubes operated at 182 kv. pk., and 25 ma., with an equivalent filtration of 0.2 mm. of copper. When the tubes were brought very close together (position A), which was the position used for all experiments, intensity was 6300 r per minute. Not only were the tubes water-cooled, but an electric fan was directed upon them, and the irradiated materal was surrounded by an ice chamber. Temperature determinations were made by the use of a thermo-junction and galvanometer. The junction was placed directly into the control irradiation chamber; thus it was possible to determine the small temperature changes—which proved to be insignificant—during the entire time specimens were irradiated. Most of the irradiation work was done at a temperature of 15° C.

Although different species of *Paramecium* were irradiated and results indicated species differences in regard to x-ray susceptibility, the results reported here are based upon the use of *P. caudatum*.[3] Cultures were begun with a single specimen and cultivated in covered flasks containing either lettuce or hay infusions which were inoculated with the bacterium *Aerobacter aerogenes* as the food source.

[3] The original strain of *Paramecium caudatum* (57–14) was kindly supplied by Dr. Lauren C. Gilman, University of Miami.

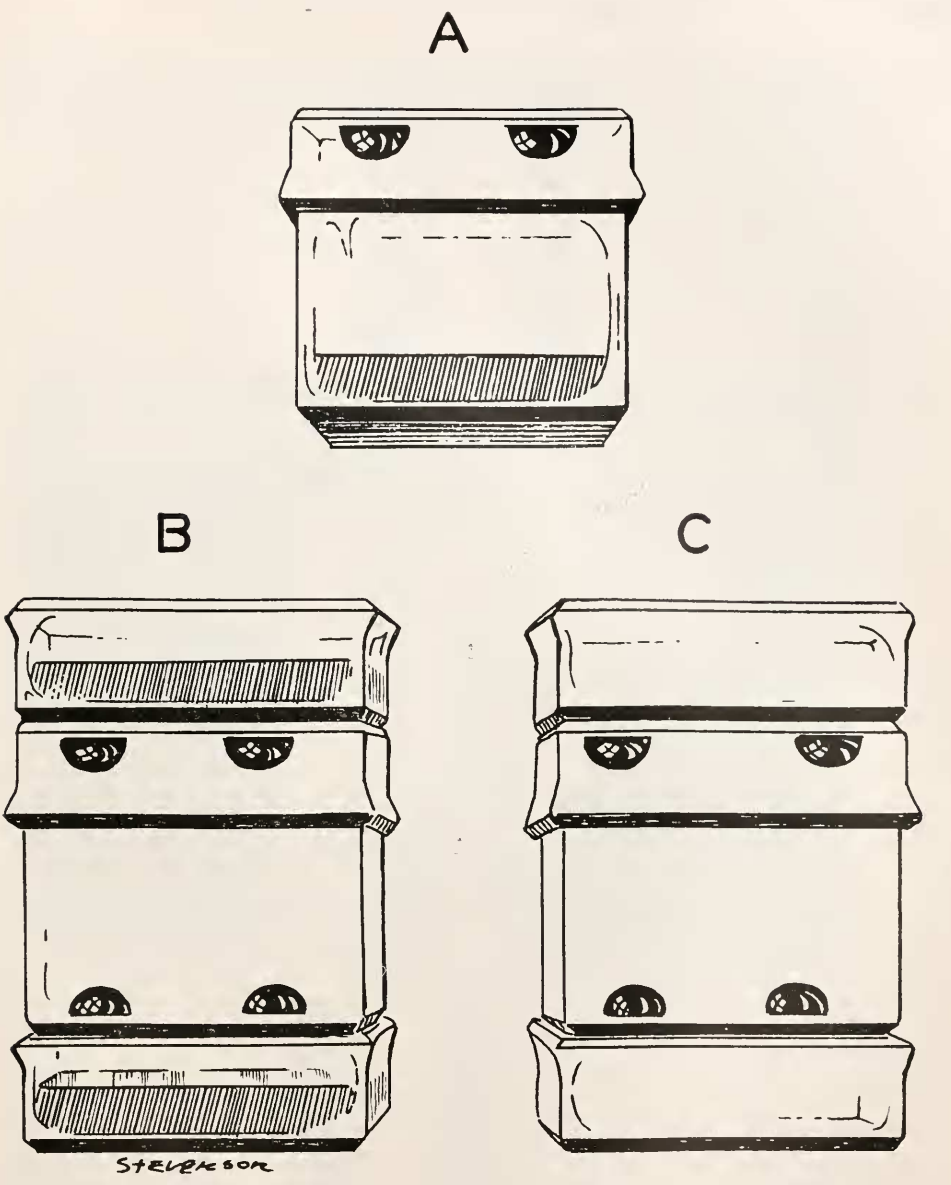

FIGURE 1. Drawings illustrating how conventional plastic boxes were used to irradiate *Paramecium caudatum* in drops and larger volumes of fluid.

Usually vegetative specimens to be irradiated were removed with a micropipette from rich clonal cultures of pH 7.1 following the logarithmic growth phase. Such active and vigorous animals were commonly uniform in size and shape. The environmental culture fluid to be irradiated with the paramecia contained fewer bacteria than during the active growth phase.

For most of the investigations, two types of irradiation chambers were employed. At first, the chambers used consisted of rigid, transparent, plastic boxes with tightly fitting lids and of a type commonly used in such experiments with microorganisms. The boxes measured approximately 24 × 24 × 18 mm. with a volume of about 6 cc. (Fig. 1, A). It was possible to irradiate four boxes containing paramecia at one time. To study the influence of the ratio of the numbers of animals to volume of fluid, drops of uniform size were suspended as hanging drops from the lids of the boxes. The drops, each containing 10, 25, 50 and 100 paramecia, were then irradiated. Additional variations were made utilizing the plastic boxes as shown in Figure 1 and described later. Subsequent experiments indicated that the number of paramecia per unit of volume was not as important in determining the lethal effects of x-radiation as the depth of the exposed culture medium, volume of the moist air-space, and the amount of surface of the culture medium exposed to the air in the radiation chamber.

A new type of radiation chamber was therefore employed to avoid the complicating factor of the air-space which appeared to diffuse from the moist air and which appeared to be extremely lethal to paramecia (Fig. 2). This new chamber consists of a Nylon hypodermic syringe of 2 cc. capacity and graduated in units of one-tenth of a cc. (0.1 cc.). A tightly fitting Lucite cap is applied over the tapering tip of each syringe. The syringe absorbs very little irradiation, eliminates air from the irradiation chamber, and permits the introduction of various substances to be tested during irradiation. The syringes may be sterilized in an autoclave. Accurate sampling of specimens after intervals of irradiation without changing the depth of the medium is also a desirable feature. A Plexiglas holder [4] measuring 11.5 × 8.5 × 2.5 cm. was designed to hold four syringes, all of which could be irradiated at the same time. The syringe-chamber method is thus ideal for the study of lethality of x-rays in *Paramecium* and should prove to be useful for similar studies with other microorganisms. Before sampling and immediately after irradiation, the syringe was quickly rotated between the fingers of both hands in order to distribute the paramecia uniformly. Usually 100 specimens in two cc. of fluid were placed in each syringe and irradiated in steps of 20,000–50,000 r. By expressing 0.2 cc. of irradiated fluid after a given dosage, it was possible to deliver into sterile Pyrex spot plates a precisely countable number of specimens—commonly ten—for the establishment of survival curves. Animals were examined immediately after irradiation, then placed in moist chambers for subsequent observation.

Results and Discussion

Irradiation with x-rays markedly increases the viscosity of the protoplasm of *Paramecium caudatum*; greater dosages lead to irreversible coagulation. Prior to

[4] The Plexiglas syringe holder with self-contained ice chambers was constructed by Mr. Michael Troisi, Instrument Maker, Temple University.

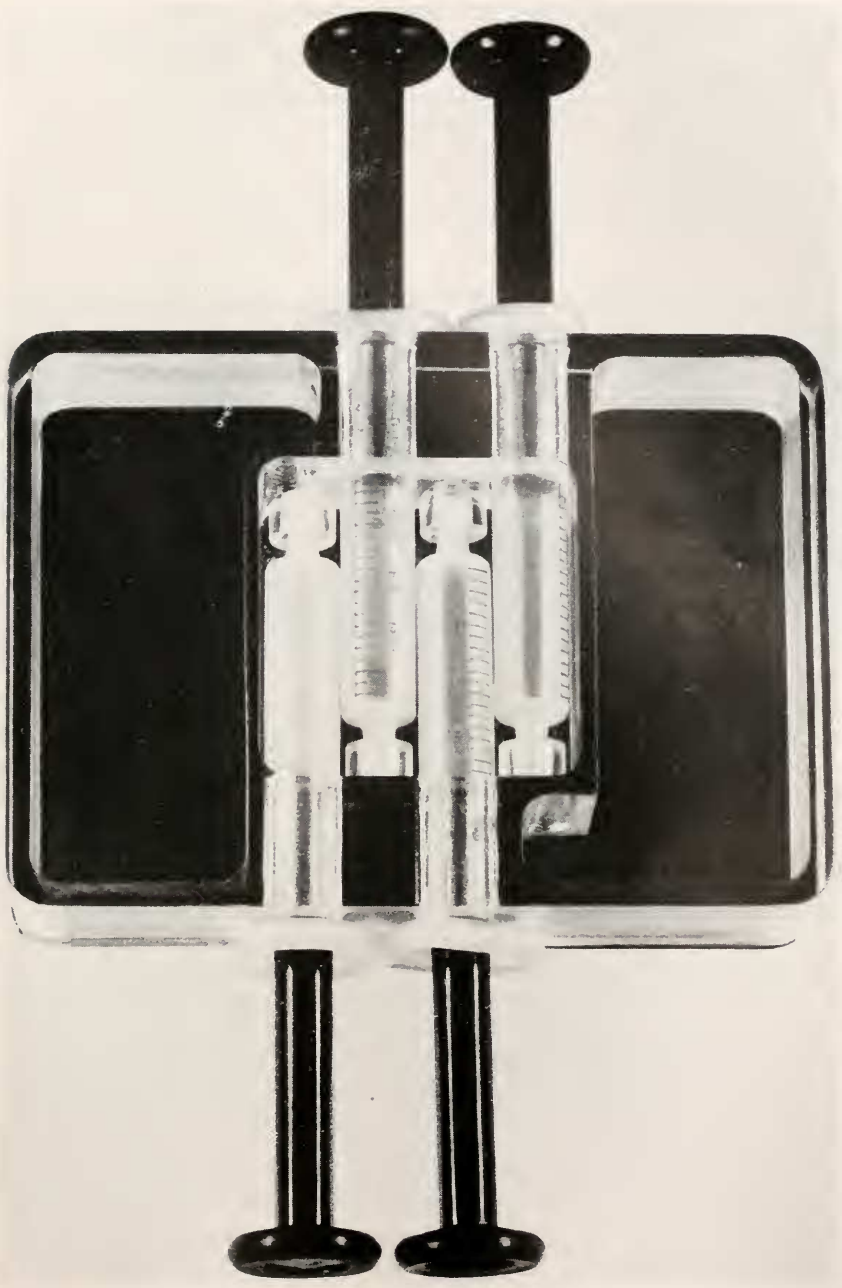

FIGURE 2. Photograph showing four 2-cc. Nylon syringes (with Lucite caps in place) being used as irradiation chambers. An ice well is present on each side of the syringe holder. (Slightly less than actual size.)

death, paramecia become immobilized, change shape to become broadly ellipsoidal and settle on the bottom of the irradiation chamber. Contractile vacuoles function more slowly and sometimes become abnormally large. Active cyclosis ceases as the protoplasm becomes conspicuously darker and vacuolated. Clear, transparent, structureless, blister-like swellings appear on the pellicle prior to death. Near death, waves of trichocysts are extruded, suggesting that these structures—commonly thought of as organelles of defense—represent a response to an injury reaction. Specimens frequently become sub-spherical before their disintegration (Fig. 3).

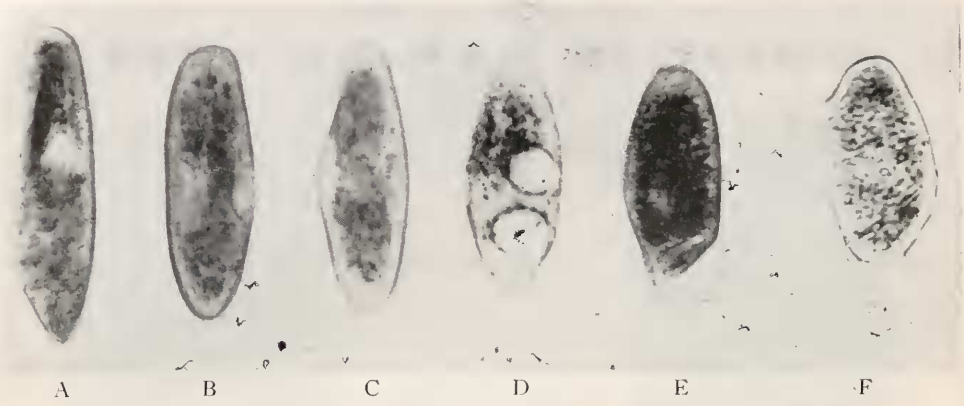

FIGURE 3. Effects of high dosage x-radiation on *Paramecium caudatum* (× 190). A: Unirradiated control specimen. B: Irradiated with 255,000 r resulting in slight change of body shape; animals generally recover from this dosage. C: Irradiated with 340,000 r (approximately the LD 50 dosage) in which locomotion and cyclosis are retarded. D: Irradiated with 425,000 r in which body shape becomes broadly ellipsoidal; greatly decreased locomotion; vacuolization. E and F: Irradiated with 510,000 r resulting in cessation of locomotion and cyclosis, increased vacuolization, blistering of pellicle, darkening (coagulation) of protoplasm followed by disintegration and death. (Photographs taken of specimens irradiated in Nylon syringes immediately after removal from x-ray generator.)

Our data are based on specimens observed for at least 24 hours after irradiation, commonly longer. The survival curves based upon this method are sigmoid as is the case with most irradiated biological material. Occasionally the slope of the curve is so steep approaching lethality as to be almost vertical. For a 24-hour period, the LD 50 for *Paramecium caudatum* is approximately 340,000 r (Fig. 4). It was soon found that the I. L. D. (immediate lethal dose), as defined by Back and Halberstaedter (1945)—that dosage which produced a complete cessation of motility within 10–15 minutes after irradiation—was not reliable as a useful endpoint. We have found that such immobilized paramecia may appear to be dead, but if examined hours later may be seen to be not only as active as control specimens but may eventually divide and produce successful clones. However, it is of interest to note that Back and Halberstaedter report the I. L. D. to be approximately 350,000 r, a dosage close to our results when using the syringe method.

The results showing percentage survival after irradiating paramecia in drops and larger volumes of fluid in plastic boxes (Fig. 1) and in Nylon syringes are

TABLE I

Survival of Paramecium caudatum after roentgen irradiation in plastic boxes and nylon syringes

Influence of the degree of exposure of animals and culture medium to air during irradiation

Type of chamber	No. of dosage groups	No. of animals observed	Per cent survival dosage in kr.									
			85	128	170	212	255	300	340	383	425	510
Paramecia in hanging drops in 6-cc. plastic boxes containing 1 cc. of culture fluid	4	55			0	0	0	0	0	0		
	12	260			0	0	0	0	0	0		
	12	140			5		0		0			
Paramecia in 1 cc. of culture fluid in bottom of plastic boxes with cover (volume 6 cc.)	10	1000	100									
	4	400			100			0				
	12	335	100		100		0		0		0	0
Paramecia in 1–2 cc. of culture fluid in Nylon syringe (no air bubbles)	36	1335	100	100	95	94	81	57	44	19	2	0

given in Table I. From this tabulation, it may be seen that the paramecia in hanging drops in plastic boxes were much more sensitive to roentgen radiation than the paramecia in the one cc. of culture fluid placed in the bottoms of the plastic boxes (Fig. 1, A). Dosages of 170 kr. killed nearly all of the paramecia in the drops whereas such dosages failed to kill any of the paramecia in the one cc. of fluid in the bottom of the plastic boxes. In most instances, the paramecia placed in hanging drops in the covers of the plastic boxes and the paramecia in the culture fluid in the bottom of the boxes were irradiated simultaneously. Variations in the concentration of paramecia in the drops and in the culture fluid in the bottom of the box did not alter this great difference in radiation sensitivity between drop and culture fluid in the bottom of the box. The only essential difference between these two conditions was the difference in the relative amount of surface exposed to air in the chambers.

It was also quite apparent that even the paramecia in the culture fluid in the bottom of the boxes succumb to the radiation in an almost "all or none" manner. When a dose of 170–200 kr. was exceeded, all paramecia died; in lower dosages, all lived. Some experiments were performed in which the influence of the depth (volume) of the culture medium was tested, since it was thought that variations in culture medium might have been responsible for an x-ray filtration effect. This did not appear to be the reason, however, for the differential sensitivity in drops, as compared with sensitivity in larger volumes of culture fluid. In some experiments, drops with 5–20 paramecia were placed in plastic boxes and one cc. of culture fluid containing 100 paramecia was placed in inverted lids above and below the plastic box chamber containing the drops (Fig. 1, B). The two cc. of culture fluid in the lids thus partially shielded the paramecia in the drops in the boxes. In other similar boxes containing drops with 5 and 20 paramecia per drop, the one

cc. of culture medium above and below was omitted (Fig. 1, C). Both sets of boxes were irradiated with 170 kr. This dose killed all of the paramecia in the drops in both boxes. All of the paramecia in the one cc. of culture fluid in the inverted lids survived. In the case of the paramecia in the inverted lid on top of the box (uncovered and exposed to the atmospheric oxygen at the surface of the culture medium), survival was 100 per cent. Thus it was apparent that all the paramecia in the drops in the plastic container were killed even though they were partially shielded by two cc. of culture fluid (one cc. above and one cc. below).

Because of this differential sensitivity resulting from differences in the degree of exposure of the culture medium to air, the plastic boxes and hanging drops were abandoned and the Nylon syringes were utilized as x-radiation chambers for the reasons given earlier. The irradiation of paramecia in the syringes (which contained no air bubbles) yielded results that were much more uniform. Using the syringe method, the results of seven experiments involving nine different dosage groups and 36 determinations are shown in Table I and the survival curve of Figure 4. In the Nylon syringes, most of the paramecia survived a dose of 170–212 kr. (lethality = 5–6 per cent). As this dosage is exceeded, however, the per cent of animals that survive 24 hours after irradiation takes a sharp drop. Generally no animals survived a 510 kr. dose.

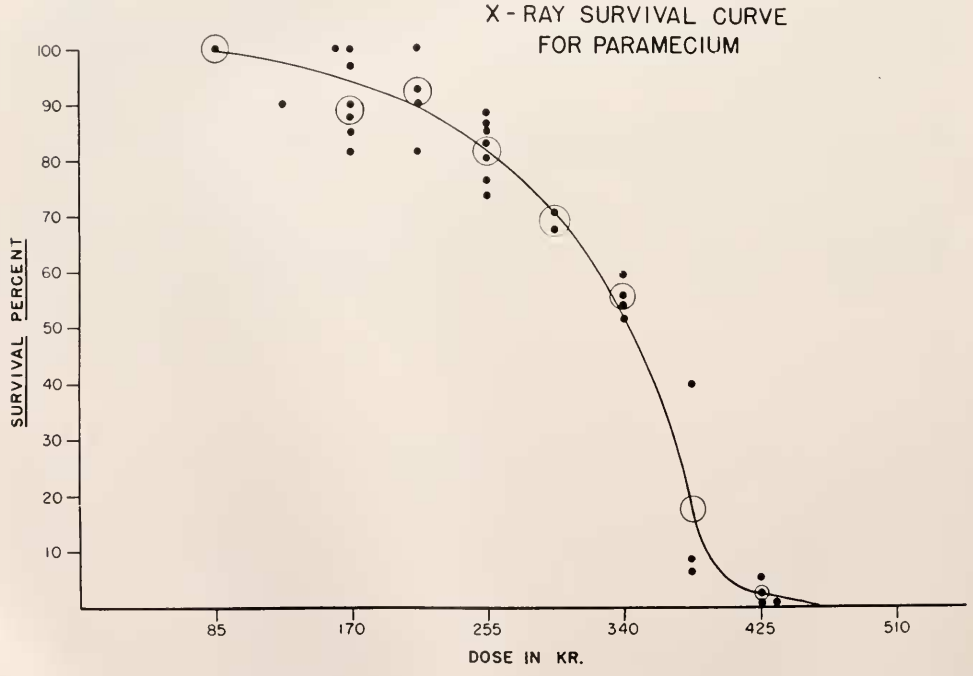

FIGURE 4. X-ray survival curve for *Paramecium caudatum* irradiated in Nylon syringes. This curve is based on seven experiments and after irradiated paramecia were observed for 24-hour period after expulsion from Nylon syringes. Coincident points are not indicated. Each point represents observations on 10–25 counted paramecia.

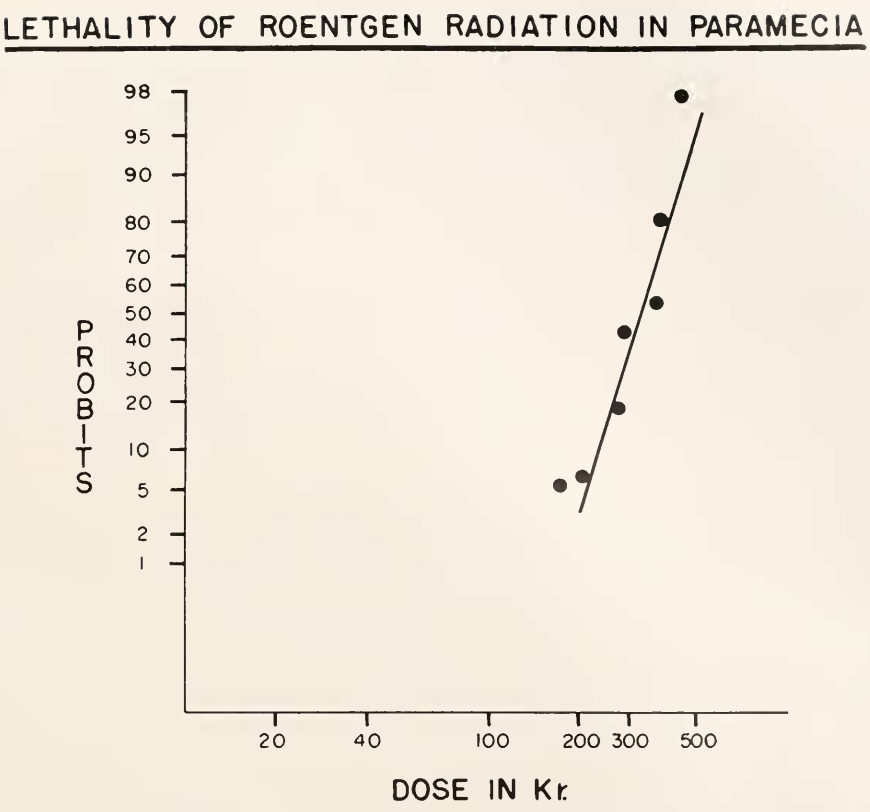

FIGURE 5. Dosage-effect curve for lethality of roentgen radiation in *Paramecium caudatum*. Results were recorded on probability paper for plotting percentages directly on a probit scale.

The data for the per cent survival after irradiation in Nylon syringes were also plotted on log-probit paper (Fig. 5). The significance of such a curve to assist in the analysis of data concerned with all-or-none responses is described by Bliss (1952). From this curve, it may be seen that while there was not a straight line relationship at the higher and lower percentages, one was present in the important range between 10 and 90 per cent. From an examination of this curve, it may be concluded that the LD 50, 24 hours, for *Paramecium caudatum* irradiated in Nylon syringes is approximately 340 kr.

From the experiments with plastic boxes, it was concluded that the number of paramecia per unit of volume was not as important in determining the lethal effects of x-radiation as the depth of the exposed culture medium, volume of the moist air-space, and the amount of surface of the culture medium exposed to the air in the irradiation chamber. This gave rise to the hypothesis that some toxic gaseous substance, possibly ozone (Taylor, 1935) was diffusing into the fluid from the irradiated moist air-space of the chamber. However, we were unable to detect ozone formation in the irradiated air of the chamber, even with the most sensitive tests. The toxic factor derived in whole or in part from the moist air in the closed boxes

during irradiation is probably oxygen or a derivative of oxygen, hydrogen peroxide or some other oxidation product.

When sealed Nylon chambers of air are irradiated with 400 kr. and unirradiated paramecia then drawn into such chambers without outside air being permitted to enter, the paramecia live for as long a period of time as the controls. This shows conclusively that the irradiated air by itself is not toxic to the animals. Also when unirradiated paramecia are placed in irradiated fluid (400 kr.) exposed and not exposed to air, and in irradiated mixtures of air and culture fluid, paramecia are not killed.

It has been known for a long time that water exposed to ionizing radiations forms hydrogen peroxide which may be lethal to ciliates (Taylor, Thomas and Brown, 1933). This does not hold for oxygen-free pure water in which no hydrogen peroxide can be demonstrated even photocolorimetrically (sensitivity 0.1 γ per ml.) (Bonét-Maury, 1951). In irradiation chambers containing clear culture fluid with bacterized paramecia, minute amounts of the enzyme catalase originate from the microorganisms and tend to offset the toxic effect of hydrogen peroxide. According to Dale (1951), one molecule of catalase can decompose 5,000,000 molecules of hydrogen peroxide per minute at 0° C. Kimball and Gaither (1952, 1953), using *Paramecium aurelia*, report that hydrogen peroxide is of major importance in the production of certain kinds of nongenetic effects but only under certain circumstances.

A study of the biological effects of ionizing radiations upon *Paramecium* must take into account the effect of these radiations on the environment in which these organisms live. The culture fluid in which the specimens are irradiated consists mainly of water with organic matter from the hay or lettuce infusions. A great body of literature demonstrates that as a result of irradiation of water, hydrogen peroxide, hydrogen and oxygen are formed in which the amounts and relative proportions depend upon such factors as dissolved oxygen concentration, radiation ionic density, dose, temperature and pH. Water that is irradiated oxidizes reducing agents and reduces oxidizing agents (Bonét-Maury, 1951).

In the irradiation of paramecia, another factor that plays a part besides the effect of ionizing radiations of water on the cell is the effect of the accompanying x-rayed bacteria present in the culture as the food source. Experiments in which the irradiated bacteria of paramecia cultures were plated out at intervals up to 350 kr. show the bacteria to have a far lower LD 50 than the paramecia. Another factor to take into account is the indirect or direct effect of radiations of the dead bacteria and their fragmented cells upon paramecia. The experiments with bacteria also showed the necessity of bacterizing spot plates containing irradiated paramecia and fluid if one is to make observations over long periods of time. Failure to do this will result in slower division rates; perhaps ultimate starvation of the paramecia in irradiated paramecia samples.

Summary

1. Irradiation with x-rays markedly increases the viscosity of the protoplasm of *Paramecium caudatum*; greater dosages lead to irreversible coagulation. With increased irradiation, paramecia become immobilized, become broadly ellipsoidal and settle on the bottom of the irradiation chambers. Contractile vacuoles function

more slowly and occasionally become abnormally large. Prior to death, cyclosis ceases and the protoplasm becomes darker and vacuolated. Clear, blister-like swellings appear at the pellicle. Before death, waves of trichocysts are extruded suggesting that their function may represent an injury-reaction. Finally, paramecia frequently become sub-spherical before their disintegration.

2. It was found that one of the most important factors influencing the lethal effects of x-radiation was the degree and extent of exposure of the fluid containing paramecia to air. Paramecia in hanging drops were killed by dosages (170 kr.) that exhibited no lethality for paramecia in larger volumes of culture fluid. This difference in lethality occurred even though the numbers of paramecia per unit volume were kept uniform in both drops and larger volumes.

3. A new method using Nylon syringes was devised to minimize the variability of x-radiation effects.

4. Survival curves were established for *Paramecium caudatum* using this new method. It was found that the LD 50, 24 hours was approximately 340 kr.

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